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A cationic liposome–DNA complexes adjuvant (JVRS-100) enhances the immunogenicity and cross-protective efficacy of pre-pandemic influenza A (H5N1) vaccine in ferrets

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Abstract

Influenza A (H5N1) viruses continue to pose a public health threat. As inactivated H5N1 vaccines are poorly immunogenic, adjuvants are needed to improve the immunogenicity of H5N1 vaccine in humans. Here, we investigated the immunogenicity and cross-protective efficacy in ferrets of a clade 2.2-derived vaccine with addition of JVRS-100, an adjuvant consisting of cationic liposome–DNA complexes (CLDC). After the first vaccination, significantly higher levels of hemagglutination-inhibition (HAI) and neutralizing antibody titers were detected in ferrets immunized with adjuvanted vaccine compared to unadjuvanted vaccine. Following a second dose of adjuvanted vaccine, HAI antibody titers of 40 were detected against viruses from multiple H5N1 clades. HAI antibodies against newly isolated H5N2 and H5N8 viruses were also augmented by JVRS-100. Ferrets were challenged with a heterologous H5N1 virus. All ferrets that received two doses of adjuvanted vaccine exhibited mild illness, significantly reduced nasal wash virus titers and protection from lethal challenge. In contrast, ferrets that received unadjuvanted vaccine showed greater weight loss, high viral titers and 3 of 6 animals succumbed to the lethal challenge. Our results indicate that the addition of JVRS-100 to H5N1 vaccine enhanced immunogenicity and cross-protection against lethal H5N1 virus disease in ferrets. JVRS-100 warrants further investigation as a potential adjuvant for influenza vaccines.

Keywords

Influenza A (H5N1) vaccine; CLDC adjuvant; Antibody response; Cross-protection; Ferrets

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Conflict of interest

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5. Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

1. Introduction

Influenza is an acute respiratory disease caused by infection with influenza viruses (Centers for Disease Control and Prevention, 2010). Seasonal influenza viruses that circulate annually in humans including influenza A (H3N2 and H1N1 subtypes) and B viruses result in more than 200,000 respiratory and circulatory hospitalizations each year, and an average of 23,000 annual influenza-associated deaths in the United States (Centers for Disease Control and Prevention, 2010; Thompson et al., 2004). With the implementation of influenza vaccination programs, illness and hospitalizations associated with seasonal influenza virus have been substantially averted in the United States (Kostova et al., 2013). However, there are far fewer licensed pre-pandemic vaccines available for use against other influenza A subtypes such as avian influenza A (H5N1) virus that has caused sporadic human infections since 1997 (WHO, 2014) and A (H7N9) that have caused outbreaks of human illness in China since February 2013 (Gao et al., 2013). The continuing occurrence of avian influenza virus infections in humans and the suboptimal immunogenicity of vaccines that target them, highlight the need for the development of improved vaccines strategies (Bart et al., 2014; Prieto-Lara and Llanos-Mendez, 2010; Treanor et al., 2006).

Highly pathogenic avian influenza (HPAI) A (H5N1) virus was first isolated from humans in Hong Kong in 1997 (Subbarao et al., 1998), and continues to cause outbreaks in poultry and sporadic human infections (WHO, 2012). Continued evolution of HPAI H5 virus has led to the emergence of 40 phylogenetically distinct HA clades of the virus (Smith et al., 2015). Viruses from clades 0, 1, 2, and 7 have caused human disease (Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus et al., 2008), but clade 1 and clade 2 viruses are responsible for recent human infections (WHO, 2015b). Between 2003 and November 2015, the World Health Organization (WHO) reported 844 laboratory confirmed human cases of H5N1 virus infection with a case fatality rate of approximately 53% (WHO, 2015a). Recent studies suggest that H5N1 virus have the potential to acquire airborne transmission in mammals (Herfst et al., 2012; Imai et al., 2012). Furthermore, humans are largely serologically naive to the H5N1 subtype based on the available H5N1 seroepidemiologic studies (Liao et al., 2013; Nasreen et al., 2013; Van Kerkhove, 2013). In addition, newly isolated HPAI H5 viruses have recently caused outbreaks in wild birds and domestic poultry in Asia, Europe, and North America (Jhung et al., 2015; Smith et al., 2015; WHO, 2015b). Although human infections caused by these viruses have not been detected to date, their appearance in North America highlights the intercontinental spread of H5 virus, the genetic reassortment with regionally circulating avian influenza viruses, and collectively, represents a significant threat to human health (Smith et al., 2015).

As vaccination is one of the most effective strategies for prevention and control of seasonal and pandemic influenza, H5 vaccines have been developed and evaluated intensively in both preclinical studies in mice or ferrets, and in humans (Baras et al., 2008a, 2008b; Baz et al., 2013; Belshe et al., 2014; Leroux-Roels and Leroux-Roels, 2009; Subbarao and Luke, 2007). In preclinical and clinical trials, inactivated H5N1 vaccines were consistently poorly immunogenic when administered to animals or human volunteers without adjuvant. The addition of proprietary oil-in-water emulsion adjuvants such as AS03 and MF59 to

inactivated H5N1 vaccines has enhanced vaccine immunogenicity and cross-clade reactivity (Baras et al., 2008a, 2008b; Belshe et al., 2014; Bihari et al., 2012), but predominantly stimulate humoral immune responses; the role of cellular immune responses in post-vaccination protection has not been clearly elucidated (Leroux-Roels, 2009; Radosevic et al., 2008; Valensi et al., 1994). Previous murine studies showed that squalene-based MF59 adjuvant primarily enhances the Th2/IgG1 response to inactivated influenza vaccine and has little effect on the Th1/IgG2a response that is superior in providing protection against infections from antigenically drifted or shifted influenza viruses in mice (Moran et al., 1999; Valensi et al., 1994). Given the rapid antigenic evolution of H5 virus, the use of an adjuvant promoting Th1 response result in both humoral and cell-mediated immune responses that may lead to better cross-protection against heterologous H5 virus infection (Thomas et al., 2006).

JVRS-100 is comprised of cationic liposome–DNA complexes (CLDC) and was developed as an adjuvant for vaccines against infectious diseases and as an immunomodulator for cancer therapy (Bernstein et al., 2009; Chang et al., 2009; Lay et al., 2009; Zaks et al., 2006). It has been demonstrated that the addition of CLDC to influenza vaccines can induce robust CD4⁺ Th1 response and predominant IgG2a/c antibody responses in mice (Chang et al., 2009; Dong et al., 2012; Hong et al., 2010; Lay et al., 2009). Most noteworthy, enhanced multicytokine-producing influenza virus-specific CD4⁺ and CD8⁺ T-cell responses were demonstrated in mice and non-human primates, and enhanced cross-protection were demonstrated in mice with addition of CLDC to influenza vaccines (Hong et al., 2010; Lay et al., 2009). In a previous study, we demonstrated that a JVRS-100 adjuvanted clade 1 H5N1 split vaccine showed significantly improved immunogenicity and antigen-sparing effect, stimulated enhanced cross-clade antibody and CD4⁺ T-cell responses, and provided significant cross-clade protection in mice (Dong et al., 2012). Ferrets have been widely used and recognized as the most suitable pre-clinical challenge model to evaluate the immunogenicity and protective efficacy of influenza vaccines (Baras et al., 2008b; Subbarao and Luke, 2007; Zitzow et al., 2002). Therefore, pre-clinical data generated in this species could be predictive of clinical trials in humans when it is not possible to conduct experimental challenge of HPAI H5 virus in human volunteers (Subbarao and Luke, 2007). Here, we investigated the immunogenicity of a JVRS-100 adjuvanted clade 2.2 H5N1 split vaccine from the US pre-pandemic influenza vaccine stockpile, and evaluated the cross-protective efficacy against infection with a heterologous H5N1 strain in ferrets.

2. Materials and methods

2.1. Viruses

HPAI H5N1 viruses used in this study included A/bar-headed goose/Qinghai/1A/2005 (BH/05), A/Indonesia/05/2005 (IN/05), A/Hong Kong/156/1997, A/Vietnam/1203/2004, A/Cambodia/X012331/2013, A/Indonesia/NIHRD/12379/2012, A/Bangladesh/3233/2011, A/Anhui/1/2005, and A/Hong Kong/6841/2010. HPAI H5N8 (A/gyrfalcon/Washington/410886/2014) and H5N2 (A/northern pintail/Washington/40964/2014) viruses were also used. Virus stocks were propagated in the allantoic cavity of 10 day-old embryonated chicken eggs, then were aliquoted and stored at –80 °C until used. Fifty percent egg-

infectious dose (EID₅₀) titers were determined by serial titration of virus in eggs (Lu et al., 1999). Virus propagation and titration were conducted under biosafety level 3 containment with enhancements (BSL-3E) required by the U.S. Department of Agriculture and the Select Agent Program to ensure the safety of laboratory workers, the environment, and the public (<http://www.cdc.gov/biosafety/publications/bmbl5/>).

2.2. Preparation of JVRS-100 adjuvanted H5N1 vaccine

JVRS-100 (Colby Pharmaceuticals, San Jose, CA) was manufactured from pDNA (pMB75.6) with DOTIM/cholesterol liposomes as previously described (Lay et al., 2009). Inactivated split egg-based BH/05 clade 2.2 H5N1 vaccine (Lot no. UD10026) was manufactured by Sanofi Pasteur and obtained through the Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority (BARDA). Vaccine potency was established by the Center for Biologics Evaluation and Research, Food and Drug Administration (CBER/FDA). The vaccine was diluted in 5% dextrose in water and then mixed with JVRS-100 prior to immunization.

2.3. Immunization of ferrets

Male Fitch ferrets, 6–8 months of age (Triple F Farms, Sayre, PA), were housed in cages within a Duo-Flo Bioclean mobile clean room (Lab Products, Seaford, DE) throughout the experiment. Ferrets were confirmed to be serologically negative by HAI assay for currently circulating seasonal influenza viruses. Baseline serum, temperature and weight measurements were obtained prior to challenge as previously described (Maines et al., 2005). Ferrets were anesthetized with an intramuscular (i.m.) injection of ketamine hydrochloride cocktail [ketamine, xylazine, and atropine]. Groups of 6 ferrets received intramuscularly 2 doses 28 days apart of one of the following: (1) JVRS-100 adjuvanted BH/05 vaccine containing 7.5 µg HA+15 µg of JVRS-100/per dose; (2) BH/05 vaccine alone (7.5 µg HA/per dose); and (3) JVRS-100 only (15 µg/per dose). The animals were injected i.m. with 250 µl of vaccine into the right hind leg muscle. Serum samples were collected on day 0 and 28 after the first immunization, 2 weeks and 2 months (pre-challenge) after the second immunization then frozen at –20 °C until testing.

2.4. Serological assays

Serum samples were treated with receptor-destroying enzyme from *Vibrio cholerae* (Denka-Seiken, Tokyo, Japan) before testing for the presence of virus-specific antibodies. The HAI assay was performed using 4 hemagglutination units (HAU) of live H5N1 virus and 1% horse red blood cells (Stephenson et al., 2004). Titers of neutralizing antibody were determined by microneutralization (MN) assay as previously described (WHO, 2011). Both HAI and MN assays were performed under BSL-3E containment.

2.5. Ferret challenge with H5N1 virus

Ferrets were challenged 2 months after the second immunization with a lethal dose (10⁶ EID₅₀) of IN/05 H5N1 virus administered intranasally (i.n.) and then monitored daily for clinical signs of infection, weight loss, and survival. Nasal washes were collected at day 2, 4,

and 6 post-infection (p.i.), and were titrated in eggs as previously described (Maines et al., 2005). Ferrets that lost more than 25% of total body weight or exhibited signs of neurological dysfunction were euthanatized. All animals used in this study were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by CDC's Institutional Animal Care and Use Committee. The challenge experiment was conducted under animal BSL-3E containment.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 5 software (Graph-Pad Software, Inc., San Diego, CA). Statistical significance of the data was determined by using *t* test, or Kaplan-Meier survival curves and log rank tests to calculate *p* values. $p < 0.05$ was considered as a significant difference.

3. Results

3.1. Immunogenicity of JVRS-100 adjuvanted clade 2.2 H5N1 split vaccine in ferrets

To evaluate whether JVRS-100 could enhance immunogenicity of an H5N1 vaccine, ferret sera were collected four weeks after the first immunization and two weeks after the second immunization. As shown in Fig. 1, ferrets that received JVRS-100 only did not exhibit any HAI or MN antibodies at any time point. Vaccine administered without adjuvant elicited little or no detectable serum HAI and MN antibodies even after two doses. In contrast, the administration of one dose of BH/05 vaccine with JVRS-100 resulted in a significantly higher response in HAI antibody titers compared to the vaccine alone group ($p < 0.01$); 50% (3/6) of the ferrets exhibited an HAI titer of 40, which is generally accepted to represent a 50% protective titer for seasonal influenza A (H3N2) viruses in adult populations (Potter and Oxford, 1979). Following the second immunization of adjuvanted vaccine, the serum antibody response was further boosted and all the ferrets achieved HAI and MN titers of 80 to the homologous virus. These results demonstrate that the JVRS-100 adjuvant significantly enhanced the immunogenicity of the clade 2.2 split vaccine in ferrets.

3.2. Cross-reactive HAI antibody responses of JVRS-100 adjuvanted clade 2.2 H5N1 split vaccine

To investigate whether the administration of JVRS-100 adjuvant to a clade 2.2 vaccine could also augment cross-clade antibody responses, sera from each group of six ferrets collected two weeks after the second immunization were evaluated by HAI assays against 11 H5 viruses isolated between 1997 and 2014 (Table 1). BH/05 vaccine alone failed to induce detectable amounts of HAI antibody to the homologous and heterologous H5 viruses. In contrast, JVRS-100 adjuvanted BH/05 vaccine not only significantly enhanced cross-reactive HAI antibody response to the 1997–2013 H5N1 viruses ($p < 0.01$), but also significantly enhanced cross-reactive HAI antibody response against heterologous 2014 H5N2 and H5N8 viruses ($p < 0.01$) compared to the vaccine only group. Collectively, our results demonstrated that co-administration of JVRS-100 with a clade 2.2 H5N1 split vaccine in ferrets can enhance HAI antibody responses to the homologous and heterologous H5 viruses isolated between 1997 and 2014.

3.3. Cross-protective efficacy of JVRS-100 adjuvanted clade 2.2 H5N1 split vaccine in ferrets

Ferret sera were collected before challenge (2 months after the second immunization) and tested for HAI and MN antibodies to BH/05 and IN/05 viruses. As presented in Table 2, no pre-challenge HAI and MN antibodies were detected in ferrets immunized with vaccine alone. In contrast, ferrets that received vaccine with JVRS-100 exhibited substantial antibody titers to BH/05 and IN/05 viruses with HAI and MN GMTs of > 40 ($p < 0.01$).

Protective efficacy against heterologous IN/05 virus was augmented by JVRS-100. As presented in Table 2, ferrets that received vaccine alone or JVRS-100 alone showed significantly higher virus titers in nasal washes on day 2, 4 and 6 p.i. compared to ferrets that received adjuvanted vaccine ($p < 0.01$). The formulation of vaccine with JVRS-100 resulted in about an 80-fold and 300-fold reduction of viral titer respectively on day 2 and 4 p.i. compared to the vaccine alone group, and complete clearance of virus in nasal washes on day 6 p.i. In accordance with the significantly reduced virus titers in nasal washes, all ferrets that received JVRS-100 adjuvanted vaccine survived a lethal challenge with a very high dose of the heterologous virus and exhibited significantly reduced morbidity as reflected by a reduction of weight loss compared to ferrets that received vaccine alone or adjuvant alone ($p < 0.01$, Fig. 2). In contrast, all ferrets that received JVRS-100 alone, and 3 of 6 ferrets that received vaccine alone, died 5–7 days p.i., These results indicate that the addition of JVRS-100 to H5N1 vaccine increased the level of cross protective immunity against a heterologous HPAI H5N1 virus challenge.

4. Discussion

HPAI clade 2.2 H5N1 viruses were first detected in migratory birds in central China in 2005, and were associated with mass die-offs of wild-birds (WHO, 2012). Since 2005, the numbers of affected countries and confirmed cases of H5N1 virus infection have increased, in part because of the spread of clade 2.2 viruses across Eurasia and to Africa (Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus et al., 2008; WHO, 2012, 2015a). In this study, we have shown that the addition of JVRS-100 to BH/05 clade 2.2-derived H5N1 vaccine, a component of the current US pre-pandemic influenza vaccine stockpile, resulted in significant enhancement of serum HAI and MN antibody responses in ferrets after the first and second vaccinations compared with unadjuvanted vaccine, and provided significant cross-protection from a lethal challenge with a highly virulent heterologous clade 2.1.3.2 virus, which has caused 199 human infections in Indonesia with very high fatality rate (84%) (WHO, 2015a). Furthermore, JVRS-100 addition also improved cross-reactive HAI antibody responses to clade 0, clade 1, clade 1.1.2, clade 2.1.3.2, clade 2.1.3.2a, clade 2.2.2, clade 2.3.4, and clade 2.3.2.1c H5N1 viruses isolated between 1997 and 2013, as well as clade 2.3.4.4 H5N2 and H5N8 viruses that emerged in late 2014. These data are consistent with our previous study showing that the addition of JVRS-100 to a licensed clade 1 H5N1 vaccine significantly enhanced cross-reactive antibody responses to clade 2.2.1 and clade 2.3.4 H5N1 viruses and provided significant cross-protections to lethal challenges with these viruses in mice (Dong et al., 2012). Importantly, JVRS-100 addition also resulted in

significant cross-clade Th1 CD4⁺ T-cell responses and dose-sparing in mice (Dong et al., 2012). The ability conferred by an adjuvant to elicit broad cross-clade protective immunity and antigen-sparing would be highly advantageous in the early stages of a pandemic to provide herd immunity in human populations, since it will take months for the current manufactures to produce well-matched vaccine with sufficient doses supply.

The addition of JVRS-100 to commercially available 2006–2007 trivalent inactivated seasonal influenza vaccine (TIV) also significantly enhanced HAI antibody responses in mice and macaques (Lay et al., 2009), and resulted in significant cross-protection in mice from antigenically distinct influenza H1N1, H3N2, and influenza B virus that were isolated from humans more than 35 years ago (Lay et al., 2009). Furthermore, JVRS-100 adjuvanted inactivated influenza H1N1 vaccine provided significant cross-protection from heterosubtypic H3N2 challenges in mice (Hong et al., 2010). Both cellular and antibody-mediated immunity likely played a role in such cross-protection from antigenically distinct influenza viruses (Hong et al., 2010; Lay et al., 2009). Occasionally, seasonal influenza vaccine antigens are antigenically drifted from circulating viruses which may in some cases lead to lower vaccine effectiveness (Flannery et al., 2015; Klimov et al., 1999; McNeil et al., 2015). The addition of adjuvants such as JVRS-100 to antigenically mismatched inactivated seasonal influenza vaccine might improve immune responses and broaden immune protection when a well-matched vaccine could not be prepared in time (Lay et al., 2009; Moran et al., 1999).

CLDC formed adjuvants such as JVRS-100 were particularly effective in eliciting strong T cell responses, even at low antigen doses (Hong et al., 2010; Lay et al., 2009; Zaks et al., 2006). The CD4⁺ and CD8⁺ T cells elicited by JVRS-100 adjuvanted influenza vaccine secrete multiple cytokines with a poly-functional phenotype in non-human primates (Lay et al., 2009). JVRS-100 adjuvanted vaccine also induced influenza virus-specific cytotoxic CD8⁺ T cells directed to the internal viral protein epitopes common to heterologous influenza strains (Hong et al., 2010; Thomas et al., 2006). Importantly, this superior cross-reactive CD8⁺ T cell reactivity induced by CLDC adjuvant persisted for long periods of time in mice lung tissues (Zaks et al., 2006), and was associated with significantly reduced morbidity and mortality in mice after heterosubtypic influenza viral challenges (Hong et al., 2010). Furthermore, increasing evidences showed that strong T cell responses to more conserved influenza internal viral proteins were associated with reduced disease severity in humans (Li et al., 2013; Sridhar et al., 2013; Wilkinson et al., 2012). Thus, influenza vaccines that can significantly boost heterosubtypic CD4⁺ and CD8⁺ T-cell responses, in addition to inducing neutralizing antibodies to the circulating viruses, may offer broader protection against drifted epidemic viruses and possibly even pandemic viruses. (Li et al., 2013; Sridhar et al., 2013; Wilkinson et al., 2012). Unfortunately, we cannot rule out a role of T cell-mediated immunity in cross-protection in this ferret model. However, it is plausible that the reduced amount of virus shedding and faster clearance of virus in ferrets might, in part, be due to a robust T cell immune response stimulated by JVRS-100 adjuvanted vaccine (Hong et al., 2010; Moran et al., 1999).

Our previous study indicated that immunization with JVRS-100 adjuvanted H5N1 vaccine induced long-lasting HAI antibody responses in mice (Dong et al., 2012). It is intriguing to

note that HAI antibody titers induced by the two doses of JVRS-100 adjuvanted H5N1 vaccine in ferrets declined 2–4 fold at 2 months compared to the time period of 2 weeks after the second immunization. The decay of the HAI antibody response was also observed in ferrets, but not in mice following immunization with alum adjuvanted H5 vaccines (Layton et al., 2011; Lu et al., 1999). The decay of the HAI antibody response was also observed in adults immunized with oil-in-water adjuvanted H5 vaccines (Leroux-Roels et al., 2010). Although antibody titers decreased with time, oil-in-water adjuvanted H5 vaccine can elicit long-term memory B cells in humans (Khurana et al., 2014) and alum adjuvanted H5 vaccine can induce long-term protection against HPAI H5N1 infection in ferrets (Layton et al., 2011). Nevertheless, successful vaccination regimes rely on the generation of long lived plasma cells and long-lived memory B cells (Tarlinton and Good-Jacobson, 2013). Investigation of immune memory enhanced by different adjuvants, including JVRS-100, may reveal mechanisms underlying the different half-lives of humoral immunity in different species. This may also lead to the development of better adjuvants that could enhance long-lasting protective immunity in humans, which is an area requiring more research.

Our data, together with other previous findings indicate that the addition of JVRS-100 adjuvant to influenza vaccines induces CD4⁺ Th1 biased immune response, cross-reactive antibody responses, functional memory CD8⁺ T-cell responses, and broad cross-protection in multiple species (Dong et al., 2012; Hong et al., 2010; Lay et al., 2009). Novel adjuvants such as JVRS-100 that can stimulate multiple immune pathways to enhance immunogenicity and protective efficacy warrant further investigation as potential adjuvants for inactivated vaccines against H5N1 and other influenza viruses with pandemic potential.

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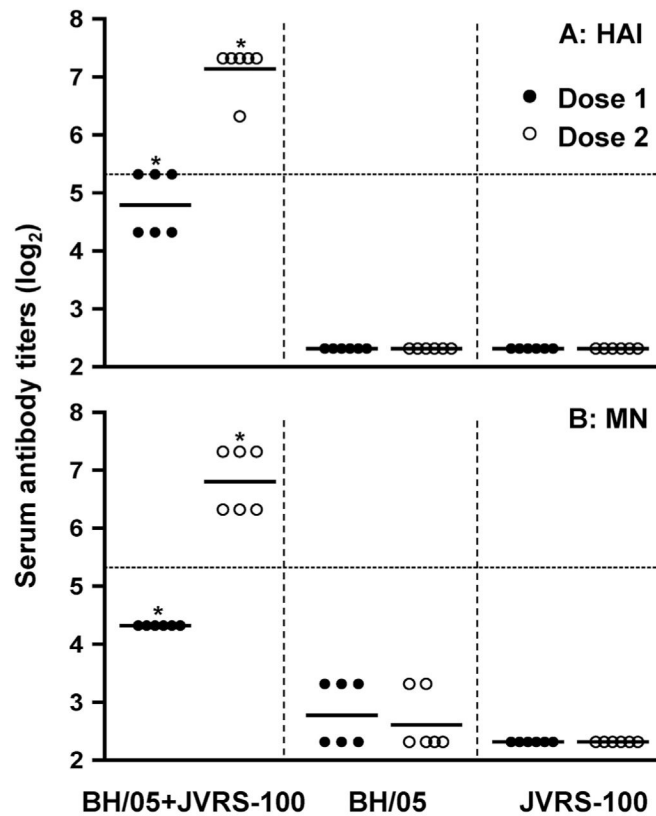
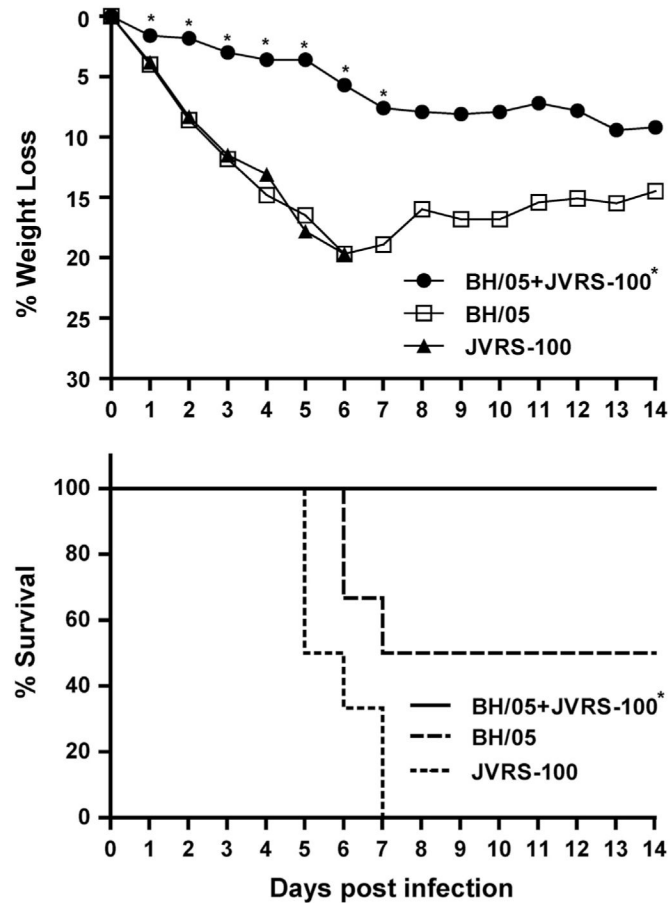


Fig. 1.

Serum HAI and MN antibody responses of ferrets immunized with BH/05 split vaccine with or without JVRS-100. Ferrets were immunized i.m with one or two doses of JVRS-100 alone (15 μ g/ferret), vaccine alone (7.5 μ g HA/ferret), or vaccine with JVRS-100 (7.5 μ g HA + 15 μ g JVRS-100/ferret). Sera were collected 4 weeks after the first dose (black dots) and 2 weeks after the second dose (open dots), and then tested individually for HAI (A) and MN (B) antibodies against the homologous virus. Antibody titers are expressed as the reciprocal of the highest serum dilution inhibiting agglutination of 1% horse erythrocytes by 4 HAU of virus, or neutralizing 100 TCID₅₀ amount of virus. Solid bars represent geometric mean titers (GMTs) of 6 ferrets per group. Dashed lines indicate the HAI and MN titer of 40. A responder is defined by neutralizing antibody titers ≥ 20 . * $p < 0.01$ compared to BH/05 vaccine alone group.

**Fig. 2.**

JVRs-100 adjuvanted clade 2.2 H5N1 vaccine significantly improves survival and morbidity against a heterologous clade 2.1.3.2 H5N1 virus infection. Groups of 6 ferrets were immunized i.m. with two doses of JVRs-100 alone (15 µg/ferret), vaccine alone (7.5 µg HA/ferret), or vaccine with JVRs-100 (7.5 µg HA+15 µg JVRs-100/ferret). Two months after the second dose, ferrets were challenged i.n. with 10^6 EID₅₀ of the heterologous IN/05 H5N1 virus. Ferrets were monitored daily for weight loss and survival for 14 days. * $p < 0.01$ compared to BH/05 vaccine alone group.

Table 1

JVRS-100 enhances serum cross-reactive HAI antibody responses in ferrets.

Influenza virus strains	Clade	Vaccine groups ^a		
		BH/05+JVRS-100	BH/05	JVRS-100
A/Hong Kong/156/1997 (H5N1)	0	50 (20–80) ^{b,*}	5	5
A/Vietnam/1203/2004 (H5N1)	1	40 (20–80) [*]	5	5
A/Cambodia/X012331/2013 (H5N1)	1.1.2	40 (20–80) [*]	5	5
A/Indonesia/NIHRD/12379/2012 (H5N1)	2.1.3.2a	71 (40–160) [*]	5	5
A/Indonesia/05/2005 (IN/05, H5N1)	2.1.3.2	113 (80–160) [*]	5	5
A/bar-headed goose/Qinghai/1A/2005 (BH/05, H5N1)	2.2	<u>143 (80–160)[*]</u>	5	5
A/Bangladesh/3233/2011 (H5N1)	2.2.2	71 (40–80) [*]	5	5
A/Anhui/1/2005 (H5N1)	2.3.4	57 (40–80) [*]	5	5
A/Hong Kong/6841/2010 (H5N1)	2.3.2.1c	40 (40–40) [*]	5	5
A/gyrfalcon/Washington/410886/2014 (H5N8)	2.3.4.4	14 (10–40) [*]	5	5
A/northern pintail/Washington/40964/2014 (H5N2)	2.3.4.4	13 (10–20) [*]	5	5

^a Groups of six ferrets were immunized i.m. with two doses of JVRS-100 adjuvanted BH/05 vaccine, BH/05 vaccine alone or JVRS-100 alone at 28 days interval.

^b Sera were collected 2 weeks after the second immunization and tested individually by HAI assays against eleven H5 viruses. GMTs of 6 ferrets per group and range of antibody titers (in parentheses) are shown here with the homologous titer underlined.

* $p < 0.01$ compared to BH/05 vaccine alone group.

Table 2
Cross-protective efficacy of influenza A(H5N1) vaccine is augmented by JVRS-100 adjuvant.

Vaccine group ^c	Pre-challenge antibody titers ^a				Mean virus titers in nasal washes ^b				No. died/Total no. ^d
	HAI		MN		Day 2		Day 4		
	BH/05	IN/05	BH/05	IN/05	Day 2	Day 4	Day 6		
BH/05+JVRS-100	57 (40–80) *	45 (20–80) *	50 (40–80) *	45 (40–80) *	5.6±1.0 *	3.7±1.0 *	<1.2 *	0/6 *	
BH/05	5	5	5	5	7.5±0.9	6.2±0.5	4.4±1.2	3/6	
JVRS-100	5	5	5	5	7.7±0.7	6.9±1.2	5.0	6/6	

* $p < 0.01$ compared to BH/05 vaccine alone group.

^aSera were collected 2 months after the second immunization and tested individually by HAI and MN assays against BH/05 and IN/05. Antibody titers are expressed as the GMT from 6 ferrets per group with titer ranges indicated in parentheses.

^bThe viral titers in nasal washes were determined 2, 4, and 6 days p.i. respectively, and expressed as mean virus titer (Log₁₀EID₅₀/ml ±S.D.) from 6 ferrets per group at 2 and 4 days p.i., or remaining ferrets at 6 days p.i. (6 ferrets in BH/05+JVRS-100 group, 5 ferrets in BH/05 group, and 2 ferrets in JVRS-100 group). The limit of virus detection was 10^{1.2} EID₅₀/ml.

^cGroups of 6 ferrets were immunized i.m. with two doses of BH/05 (clade 2.2) vaccine with or without JVRS-100, or JVRS-100 only at 28 days interval. Two months after the boost, ferrets were challenged i.n. with 10⁶ EID₅₀ of the heterologous IN/05 (clade 2.1.3.2) H5N1 virus.

^dFerrets were monitored daily for survival for 14 days.